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Application No.	Ref.	Date
04 772 194.9 - 2405	M1335 EP S3	15.11.2007
Applicant: TAKARA BIO INC.		

Communication pursuant to Article 96(2) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(1) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 78(2) and 83(2) and (4) EPC.

One set of amendments to the description, claims and drawings is to be filed within the said period on separate sheets (Rule 36(1) EPC).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Article 96(3) EPC).

Obel, Nicolai
Primary Examiner
for the Examining Division

Enclosure(s): 5 page/s reasons (Form 2906)

Registered Letter
EPO Form 2001 07.02CSX

The examination is being carried out on the **following application documents**:

Description, Pages

1-189 as originally filed

Sequence listings, Pages

1-46 as originally filed

Claims, Numbers

1-20 received on 29.08.2007 with letter of 29.08.2007

Drawings, Sheets

1 as originally filed

1. New claims 1-20 filed with your letter dated 29.08.2007 are not allowable under Article 123(2) EPC.

1.1 Claim 1 of the amended claims is describing subject matter beyond the application as filed. The claim refers to SEQ ID NO 9 to 20 and 25 or "a polypeptide comprising at least one amino acid sequence having substitution, deletion, insertion or addition of one or the plural number of amino acids in any one of said amino acid sequences wherein the polypeptide (n) has a function equivalent to that of said polypeptide (m)". The basis is claimed to be found in claims 10 and 12 of the original set of claims.

However, the variants of the sequences are described in claim 10 and are with regard to SEQ ID NO 1-8. No variants of SEQ ID NO 9 and 20-25 are disclosed in claim 12 of the original set of claims. The fact that the original claim 12 was dependent on claim 10 can not serve as a basis as SEQ ID NO 9 and 20-25 represent specific embodiments within the scope of claim 10 of the original set of claims. The amendment describes thus subject matter beyond the application as filed.

1.2 The claims 1,6,13,14 and 15 are directed to a method using "a fibronectin fragment or a mixture thereof", see also 4.2. The original set of claims were describing "fibronectin, a fragment thereof or a mixture thereof". The basis is thus disclosing a mixture of fibronectin and a fibronectin fragment. The amended set of claims can only be seen as describing a mixture of at least two different fibronectin fragments and describe thus subject matter beyond the application as filed.

2. The present application does not meet the requirements of Article 52(1) EPC, because the subject-matter of claims 1,3-10,15 and 16 is not new in the sense of Article 54(1) and (2) EPC.

2.1 It is acknowledged that the amendments to claims 1 and 15 render the subject matter novel with regard to D1,D2,D5 and D7 as a serum concentration in the range 0-4% is not disclosed in said documents. Furthermore, the protein and nucleic acid sequences disclosed in the amended claims 18-20 is not disclosed in D3 and D4.

2.2 In the letter of 29.08.07, the applicant argues that D6 does not disclose the new features in the amended claim 1 which refer to the expansion of cells and the use of fibronectin fragments. However this is not correct as D6 (col 19 l. 61- col 20 l. 45) discloses a method of growing cytotoxic lymphocytes such as LAK's in the presences of an extracellular matrix protein such as fibronectin placed on a membrane. D6 (col 31 l. 51 - col 32 l. 4) discloses also that cells were bound to a proteolytic fragment of fibronectin and the binding was inhibited by 90% by RGDS, the central binding site of fibronectin (D6, col 32 l. 65 - col 33 l. 37). The document discloses thus that the binding was restricted to distinct fragments of fibronectin. D6 states also that serum free medium can be used (col 5 l. 15-28) and that mainly CD8+ cells were generated (col 16 l. 49-61, col 26 l. 10-21, col 30 l. 23-28).

D6 is directed to a method of providing activated T-lymphocytes for use in therapy. The vague definition of the fibronectin fragments to be used in the method, which is not conform with Article 123(2) and 84 EPC, see 1.1 and 4.1, means that no limitations with regard to the indicated sequences exist beyond the fibronectin annotation. D6 anticipates thus the subject matter of claims 1,3-10,15 and 16 of the amended set of claims.

3. The present application does not meet the requirements of Article 52(1) EPC, because the subject-matter of claims 2,11-14 and 17-20 does not involve an inventive step in the sense of Article 56 EPC.

3.1 The document D6 is regarded as being the closest prior art to the subject-matter of claims 2,11-14 and 17, see 2.2.

The subject matter of claim 2 is directed to a method for providing cytotoxic lymphocytes by expanding the cells on fibronectin fragments and thereby increase the expression of the IL-2 receptor.

The subject-matter of claim 2 therefore differs from this known (D6) in that the method provides a higher expression of the IL-2 receptor.

The problem to be solved can thus be regarded at to provide an improved method for providing cytotoxic lymphocytes.

D6 already provides a method for expanding cytotoxic lymphocytes on fibronectin fragments and D5 (abstract) discloses that exposure to fibronectin increased the expression of the IL-2 receptor. The subject matter of claim 2 is thus not regarded as inventive in the sense of Article 56 EPC.

In the letter of 29.08.2007, the applicant argues that surprising effects arise from the disclosed method. These effects are a high expression of the IL-2 receptor, an increased expansion rate, increased cytotoxicity and higher ratio of CD8+ cells. If such improvements were clearly demonstrated with regard to the prior art an inventive step could be acknowledged. However, no surprising effects have been

substantiated with data, documenting said alleged effects.

3.2 D1,D5 and D7 do not anticipate claims 1 and 15 of the amended set of claims. However, the only difference to these documents and the subject matter of claim 1 is the lowering of the serum concentration from 5% to 4%. The objective problem to be solved with regard to said documents would thus be how to provide an alternative method for producing cytotoxic lymphocytes. Since it is evident for the person skilled in the art that it is beneficial for patients if less serum is used, the lowering of the serum concentration from 5% to 4% can not be considered inventive. Similar to 3.1, an inventive step with regard to said claims can be acknowledged if a surprising or unexpected effect is documented.

3.3 Claims 11-14 is directed to specific steps and concentrations of cells when culturing the cytotoxic lymphocytes. Said steps and concentrations are all normally used in the art and in view of 3.1 the subject matter is not inventive in the sense of Article 56 EPC.

3.4 Claim 17 differs from D6 in the use of retro-virus or adeno-associated virus for gene transduction. These methods for gene transduction are merely some of several straightforward possibilities from which the skilled person would select, in accordance with circumstances, without the exercise of inventive skill, in order to solve the problem posed. In view of 3.1, the subject matter of claim 17 is not inventive in the sense of Article 56 EPC.

3.5 The subject matter of claims 18-20 differs from the prior art (D4) in that only a partial sequence of the fibronectin disclosed in D4 is selected. D4 (p. 3 l. 8-22) states that also fragments extending from the core 277-577 amino acids are encompassed by D4. The subject matter of claims 18-20 is thus merely representing an arbitrary selection of the sequence disclosed in D4. No surprising or unexpected effect has been documented for this molecule with regard to other fibronectin fragments and the subject matter is thus not inventive in the sense of Article 56 EPC.

4. The application does not meet the requirements of Article 84 EPC, because claims 1,6 and 15 are not clear.

4.1 If a basis is indicated for the amended claim 1 in its present form, the applicant should notice that the claim is not clear due to the term "at least one amino acid sequence having substitutions, deletions, insertions or additions of one or more plural number of amino acids". This term is not suitable to clearly define the scope of the claim, because without definition of the length of the fragment, degree of identity and precise definition of the meant portion of the molecule this expression is absolutely vague and ambiguous. The claim encompass all fibronectin fragments regardless of the indicated sequences.

4.2 Claims 1,6 and 15 are not clear as they are directed to "a fibronectin fragment or a mixture thereof". The indicated "mixture thereof" is not clear as it can not be ascertained what the mixture consists of as only a fibronectin fragment, i.e one fragment, is indicated as being used for cell expansion.

5. The applicant is requested to file new claims which take account of the above comments. The attention of the Applicant is drawn to the fact that the application may not be amended in such a way that it contains subject-matter which extends beyond the content of the application as filed, Article 123(2) EPC.

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29. Aug. 2007

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EP 04 77 2194.9-2405
Takara Bio Inc.
Our Ref.: M1335 EP S3

Munich, August 29, 2007
SZ/AF

This is in response to the Communication pursuant to Article 96(2) EPC dated February 20, 2007.

Enclosed please find new claims 1 to 20 which should form the basis for further substantive examination. We herewith reserve our right to file divisional applications for subject matter which was deleted from the claims or which is no longer covered by the new claims.

1. AMENDMENTS IN THE CLAIMS

1.1 New claim 1 is based on original claim 1 which has been amended as follows:

(i) The term "a cytotoxic lymphocyte" has been corrected to "cytotoxic lymphocytes".

(ii) The phrase "at least one step selected from induction, maintenance and" has been deleted and the term "an" has been inserted before "expansion".

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(iii) "5% by volume" has been amended to "4% by volume".
Support for this amendment can be found, e.g. on page 22, lines 22 to 24 of the application.

(iv) The phrase "fibronectin, a fragment thereof or a mixture thereof" has been restricted to "a fibronectin fragment or a mixture thereof".

(v) Moreover, the fibronectin fragment has been further specified as one consisting of SEQ ID NOs: 9 to 20 or 25 or as a polypeptide deviation from these fragments but having a function equivalent to SEQ ID NOs: 9 to 20 or 25.
Support for this amendment can be found, e.g., in original claims 10 and 12.

1.2 New claims 2 to 5 correspond to original claims 2 to 5 with the only exception that the following amendments have been effected:

(i) The term "cytotoxic lymphocyte" has been corrected to "cytotoxic lymphocytes".

(ii) It has been clarified that the method is for preparing cytotoxic lymphocytes with the indicated activities/properties.

1.3 New claim 6 corresponds to original claim 6 which has been restricted to the fibronectin fragments mentioned in claim 1.

1.4 New claims 7 and 8 correspond to original claims 7 and 8.

1.5 New claim 9 corresponds to original claim 9 with the only exception that the singular has been corrected to plural.

1.6 Original claim 10 has been deleted.

1.7 New claim 10 corresponds to original claim 11 with the back reference adjusted.

1.8 Original claim 12 has been deleted.

1.9 New claim 11 corresponds to original claim 13.

1.10 New claim 12 corresponds to original claim 14 with the back reference adjusted.

1.11 New claims 13 and 14 correspond to original claims 15 and 16, respectively, wherein "at least any one of induction, maintenance and" has been deleted, "an" has been inserted before "expansion", "a cytotoxic lymphocyte" has been corrected to "cytotoxic lymphocytes" and the claims have been restricted to fibronecin fragments.

1.12 Original claims 17 and 18 have been deleted.

1.13 New claim 15 corresponds to original claim 19 in which the same amendments as set forth in section 1.1(i), (iii) and (iv) for claim 1 have been effected.

1.14 New claim 16 corresponds to original claim 20 in which "a cytotoxic lymphocyte" has been corrected to "cytotoxic lymphocytes" and the back reference has been adjusted.

1.15 New claim 17 corresponds to original claim 21 with the back reference adjusted.

1.16 New claim 18 corresponds to original claim 22 in which the term "having" has been replaced by "consisting of" and the phrase "of one or the plural number of amino acid(s)" has been replaced by "of 1-20 amino acids".
Support for the latter amendment can, e.g. be found on page 48, lines 1 to 5 of the application.

1.17 New claim 19 corresponds to original claim 23 with the back reference adjusted.

1.18 New claim 20 corresponds to original claim 24 with the exception of the following amendments:

(i) The "comprising" language has been replaced by "consisting" language.

(ii) The back reference has been adjusted.

(iii) The phrase "of one or the plural number of nucleotide(s)" has been replaced by "of 1-60 nucleotides".
This amendment is, e.g., supported by the disclosure content provided on page 48, last line to page 49, line 4.

(iv) Part (3) has been deleted.

For the convenience of the Examiner we enclose a copy of the claims from which the effected amendments are evident.

2. NOVELTY (ARTICLE 54 EPC)

2.1 Claims 1 and 19 (new claims 1 and 15)

(a) EP-A1 1 424 387 (D1)

In section 2.1 of the Communication original claims 1 and 19 are objected to as lacking novelty over EP-A1 1 424 387 (D1) in particular in view of the disclosure provided in paragraph 165 in combination with paragraph 77 and in view of claim 8 of D1.

This novelty objection does not apply to the new claims. It has been specified in the claims that the serum and plasma concentration in the medium is between 0% by volume and less than 4% by volume. This feature is not disclosed in D1. The medium used in D1 contains 5% serum as is evident from paragraph [0077], line 26 in connection with paragraph [0165]. Thus, new claims 1 and 15 are novel over D1.

(b) Cardarelli (D5)

In section 2.1 of the Communication original claims 1 and 19 are also objected to as lacking novelty over Cardarelli (D5), in particular the disclosure provided in the "Materials and Methods" section.

This objection does not apply to the new claims. In particular, the claims have been restricted to the use of certain fibronectin fragments. D5, however, does not disclose the use of fibronectin fragments. Thus, new claims 1 and 15 are novel over D5.

(c) US 5,354,686 (D6)

In section 2.1 of the Communication original claims 1 and 19 are also objected to as lacking novelty over US 5,354,686 (D6).

However, we submit that this novelty objection is not justified and, in particular, does not apply to the new claims for the following reasons: D6 relates to T-cells which are capable of binding to an extra-cellular matrix protein (ECM protein) such as fibronectin. However, it fails to teach or suggest effects by using fragments of fibronectin. Also, this reference merely discloses to assess the likelihood of binding of T-cells to fibronectin as ECM with serum-free cell culture medium, but it does not disclose expanding T-cells with serum-free culture medium.

Thus, new claims 1 and 15 are also novel over D6.

(d) Takashi (D7)

In section 2.1 of the Communication original claims 1 and 19 are also objected to as lacking novelty over Takashi (D7).

However, we submit that this novelty objection is not justified and does not apply to the new claims.

D7 discloses activation of T-cells by fibronectin or vitronectin. However, D7 does not disclose effects by using fragments of fibronectin. Also, serum-free medium is used in the reference only for examining SE release from cells and IL2 production. It is not disclosed to expand T-cells on serum-free medium.

Thus, new claims 1 and 15 are also novel over D7.

2.2 Original claim 17

In section 2.2 of the Communication original claim 17 is objected to as lacking novelty over Cardarelli (D5) and over US 5,354,686 (D6).

This objection does no longer apply since original claim 17 has been cancelled.

2.3 Original claims 18 and 20

In section 2.3 the Examiner also objects to original claims 18 and 20 as lacking novelty over US 5,354,686 (D6) since this document also discloses the possible use of lymphocytes in therapy and their modification by gene therapy.

As regards claim 18, this objection no longer applies since this claim has been cancelled. With respect to original claim 20 (new claim 16) the objection is not justified since this claim refers back to claim 1 which is novel over D6 as set forth in section 2.1(c), supra. Thus, the same applies to new claim 16.

2.4 Original claims 1 to 21 (new claims 1 to 17)

In section 2.4 of the Communication the Examiner cites EP-A1 1 496 109 (D2) as being novelty destroying for original claims 1 to 21. The Examiner takes the position that D2 is novelty destroying because the range of the present application is not sufficiently far from the 5% value known in the art.

This objection does not apply to the new claims because the upper level of "less than 5%" has been changed to "less than 4%".

2.5 Original claims 22 to 24 (new claims 18 to 20)

In section 2.5 of the Communication the Examiner also objects to original claims 22 to 24 as lacking novelty over WO 90/13653 (D3) and over EP-A1 207 751 (D4) arguing that certain sequences disclosed in these documents show more than 98% sequence identity to SEQ ID NO: 25 or 26.

The Applicant takes the position that this objection is not justified and does, in particular, not apply to new claims 18 to 20 which have been further specified to relate to polypeptides/nucleic acids consisting of specific sequences as defined in the claims. In this context we enclose as Annexes I and II, respectively, comparisons of SEQ ID NO: 25 and 26 with the sequences disclosed in D3 and D4, respectively.

Annex I: SEQ ID NO: 25 versus D3 (pFHDEL1)

SEQ ID NO: 25 of the present invention corresponds to only a part of the long sequence disclosed in D3. That is, as shown in Figure 1, SEQ ID NO: 25 is a fragment corresponding to the sequence of D3 positioned at 1239-1885. Accordingly, these polypeptides are quite different from each other as a substance. Further, as noted in the specification, the polypeptide of the present invention having the amino acid sequence of SEQ ID NO: 25 shows very advantageous effects in producing lymphocytes. D3 does not disclose such effects.

Annex II: SEQ ID NO: 26 versus D4 (sequence 7705)

Similar to SEQ ID NO: 25, SEQ ID NO: 26 of the present invention corresponds to only a part of the sequence disclosed in D4, and these polynucleotides are quite different from each other as a substance. Further, as noted in the specification, the polypeptide translated from the polynucleotide shows very advantageous effects in producing lymphocytes. D4 does not disclose such effects.

Thus, Applicant submits that new claims 18 to 20 are novel over D3 and D4.

3. INVENTIVE STEP (ARTICLE 56 EPC)

The claimed subject-matter also involves inventive step over the prior art.

As noted at page 5, line 10 to page 6, line 4 of the specification as "Background Art" in conventional methods for expanding large amounts of lymphocytes, culture mediums containing 5-20% by volume of serum or plasma had been used. In contrast to this, the present invention is based on the finding that the presence of certain fragments of fibronectin enables expanding cytotoxic lymphocytes at a high efficiency even with a smaller amounts of serum or plasma. The present invention is practically very advantageous in the field of cell therapy because the amount of blood from a patient in adoptive immunotherapy can be reduced by lowering the amount of serum or plasma used in the culture medium. Moreover, lymphocytes obtained by the method of the present invention highly express the IL-2 receptor, contain CD8-positive cells in a higher ration, and have higher cytotoxic activity as described at page 11, lines 7 to 13 of the specification. Thus, the present invention exhibits superior effects in the cellular medical field.

None of the above-mentioned cited references provides any hint to expand lymphocytes by lowering the amount of serum or plasma in the culture medium.
Moreover, none of the cited prior art references discloses or suggests the use of fibronectin fragments for expanding the lymphocytes.
Finally, none of the cited prior art references provides any hint that any of the above-described advantageous effects as regards the obtained lymphocytes can be obtained. Thus, taken together, it has to be concluded that the claimed subject-matter involves inventive step.

4. RULE 29(3) EPC

In section 3.1 of the Communication original claims 2 to 5 are objected to as only describing an effect.

We submit that this objection is not justified and does, in particular, not apply to the new claims.
In the new claims 2 to 5 it has been clarified that the claimed method is for preparing lymphocytes having a certain property or for achieving a higher expansion.

The indication of a purpose to be achieved in a method step cannot be regarded as being unclear. Namely, it has been accepted by the case law of the Technical Boards of Appeal that a new purpose of a use (e.g. obtaining a new hitherto unrecognized effect) is a technical feature and can confer novelty and inventive step. Moreover, that the indicated effects can indeed be achieved by the claimed method is evident from the specification, e.g., page 11, lines 7 to 13 and the Examples.

5. Requests

With the above explanations and the proposed amendments to the claims, it is submitted that the Applicant has met the requirements as set forth in the Official Communication.

If, however, the Examining Division does not agree to the above, it is requested that either a further Communication pursuant to Article 96(2) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the telephone.

Dr. Friederike Stolzenburg
European Patent Attorney

Encls.
New claims 1 to 20
Copy of the claims with amendments indicated
Annexes I and II

EP 04 77 2194.9-2405
Takara Bio Inc.
Our Ref.: M1335 EP S3

Amended Claims

1. A method for preparing cytotoxic lymphocytes characterized in that the method comprises the step of carrying out an expansion of cytotoxic lymphocytes using a medium containing serum and plasma at a total concentration of 0% by volume or more and less than 4% by volume, in the presence of a fibronectin fragment or a mixture thereof,
wherein the fibronectin fragment is at least one polypeptide (m) selected from the group consisting of polypeptides having any one of the amino acid sequences shown in SEQ ID NOs: 9 to 20 and 25 of Sequence Listing, or a polypeptide comprising at least one amino acid sequence having substitution, deletion, insertion or addition of one or the plural number of amino acids in any one of said amino acid sequences, wherein the polypeptide (n) has a function equivalent to that of said polypeptide (m).

2. The method according to claim 1, wherein the method is for preparing cytotoxic lymphocytes which highly express an interleukin-2 receptor as compared to cytotoxic lymphocytes prepared in the absence of fibronectin, a fragment thereof or a mixture thereof.

3. The method according to claim 1, wherein the method is for preparing cytotoxic lymphocytes which contain CD8-positive cell in a higher ratio as compared to cytotoxic lymphocytes prepared in the absence of fibronectin, a fragment thereof or a mixture thereof.

4. The method according to claim 1, wherein the method is for being higher expansion fold as compared to that of a method for preparing cytotoxic lymphocytes in the absence of fibronectin, a fragment thereof or a mixture thereof.

5. The method according to any one of claims 1 to 4, wherein the method is for preparing cytotoxic lymphocytes the cytotoxic activity of which is enhanced or high cytotoxic activity is maintained as compared to a cytotoxic activity of cytotoxic lymphocytes prepared in the absence of fibronectin, a fragment thereof or a mixture thereof.

6. The method according to any one of claims 1 to 5, wherein the fibronectin fragment or a mixture thereof is immobilized on a solid phase.

7. The method according to claim 6, wherein the solid phase is a cell culture equipment or a cell culture carrier.

8. The method according to claim 7, wherein the cell culture equipment is a petri dish, a flask or a bag, and the cell culture carrier is beads, a membrane or a slide glass.

9. The method according to any one of claims 1 to 8, wherein the cytotoxic lymphocytes are lymphokine-activated killer cells.

10. The method according to any one of claims 1 to 9, wherein the fibronectin fragment has a cell adhesion activity and/or a heparin binding activity.

11. The method according to claim 1 which is carried out in a cell culture equipment, wherein the method satisfies the conditions of:
    (a) a ratio of the number of cells to a culture area in the cell culture equipment at initiation of culture being 1 cell/cm^2 to 5 X 10^5 cells/cm^2 ; and/or
    (b) a concentration of cells in a medium at initiation of culture being 1 cell/mL to 5 X 10^5 cells/mL.

12. The method according to claim 11, wherein the method does not require a step of diluting a cell culture solution.

13. The method according to claim 1, wherein the method comprises carrying out an expansion of cytotoxic lymphocytes in the presence of the fibronectin fragment or a mixture thereof in a cell culture equipment containing a medium, wherein the method comprises at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment, and wherein the culture conditions immediately after at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment satisfy the conditions of:

(c) a concentration of cells in the cell culture solution being 2 X 10^5 cells/mL to 1 X 10^8 cells/mL; or

(d) a ratio of the number of cells in the cell culture solution to a culture area in the cell culture equipment being 1 X 10^5 cells/cm^2 to 1 X 10^8 cells/cm^2 .

14. The method according to claim 1, wherein the method comprises carrying out an expansion of cytotoxic lymphocytes in the presence of the fibronectin fragment or a mixture thereof in a cell culture equipment containing a medium, wherein the method comprises at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment, and wherein a total concentration of serum and plasma in the medium immediately after at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment is same as that at initiation of the culture or lowered as compared to that at initiation of the culture.

15. A medium for culturing cytotoxic lymphocytes, characterized in that the medium comprises as an effective ingredient the fibronectin fragment or a mixture thereof, and that a total concentration of serum and plasma in the medium is 0% by volume or more and less than 4% by volume.

16. The method according to any one of claims 1 to 14, further comprising a step of transducing a foreign gene into cytotoxic lymphocytes.

17. The method according to claim 16, wherein the foreign gene is transduced using retrovirus, adenovirus, adeno-associated virus or simian virus.

18. A polypeptide consisting of the amino acid sequence (x) shown in SEQ ID NO: 25 of Sequence Listing or an amino acid sequence (y) having deletion, insertion, addition or substitution of 1-20 amino acid(s) in the amino acid sequence (x), wherein the polypeptide consisting of the amino acid sequence (y) has a function equivalent to that of the amino acid sequence (x).

19. A nucleic acid encoding the polypeptide of claim 18.

20. The nucleic acid according to claim 19, wherein the nucleic acid is (1) a DNA

consisting of the nucleotide sequence shown in SEQ ID NO: 26; or (2) a DNA consisting of a nucleotide sequence having deletion, substitution, insertion or addition of 1-60 nucleotide(s) in the nucleotide sequence shown in SEQ ID NO: 26, wherein the DNA encodes a polypeptide having a function equivalent to that of the polypeptide encoded by the DNA (1).

Amended Claim

1. A method for preparing ~~a~~ cytotoxic lymphocytes characterized in that the method comprises the step of carrying out ~~at least one step selected from induction, maintenance and~~ **an** expansion of ~~a~~ cytotoxic lymphocytes using a medium containing serum and plasma at a total concentration of 0% by volume or more and less than ~~45~~% by volume, in the presence of a fibronectin~~, a~~ fragment ~~thereof~~ or a mixture thereof,

wherein the fibronectin fragment is at least one polypeptide (m) selected from the group consisting of polypeptides having any one of the amino acid sequences shown in SEQ ID NOs: 9 to 20 and 25 of Sequence Listing, or a polypeptide comprising at least one amino acid sequence having substitution, deletion, insertion or addition of one or the plural number of amino acids in any one of said amino acid sequences, wherein the polypeptide (n) has a function equivalent to that of said polypeptide (m).

2. The method according to claim 1, wherein the **method is for preparing** cytotoxic lymphocytes **which** highly expresses~~s~~ an interleukin-2 receptor as compared to ~~a~~ cytotoxic lymphocytes prepared in the absence of fibronectin, a fragment thereof or a mixture thereof.

3. The method according to claim 1, wherein the **method is for preparing** cytotoxic lymphocytes **which** contains~~s~~ CD8-positive cell in a higher ratio as compared to ~~a~~ cytotoxic lymphocytes prepared in the absence of fibronectin, a fragment thereof or a mixture thereof.

4. The method according to claim 1, wherein **the method is for being higher** ~~an~~ expansion fold ~~is higher~~ as compared to that of a method for preparing ~~a~~ cytotoxic lymphocytes in the absence of fibronectin, a fragment thereof or a mixture thereof.

5. The method according to any one of claims 1 to 4, wherein **the method is for preparing ~~a~~ cytotoxic lymphocytes the** cytotoxic activity **of which** is enhanced or high cytotoxic activity is maintained as compared to a cytotoxic activity of ~~a~~ cytotoxic lymphocytes prepared in the absence

of fibronectin, a fragment thereof or a mixture thereof.

6. The method according to any one of claims 1 to 5, wherein **the** fibronectin~~, a~~ fragment ~~thereof~~ or a mixture thereof is immobilized on a solid phase.

7. The method according to claim 6, wherein the solid phase is a cell culture equipment or a cell culture carrier.

8. The method according to claim 7, wherein the cell culture equipment is a petri dish, a flask or a bag, and the cell culture carrier is beads, a membrane or a slide glass.

9. The method according to any one of claims 1 to 8, wherein the cytotoxic lymphocytes **are** ~~is a~~ lymphokine-activated killer cell**s**.

~~10. The method according to any one of claims 1 to 9, wherein the fibronectin fragment is a polypeptide (m) comprising at least any one of the amino acid sequences shown in SEQ ID NOs: 1 to 8 of Sequence Listing, or a polypeptide (n) comprising at least one amino acid sequence having substitution, deletion, insertion or addition of one or the plural number of amino acids in any one of said amino acid sequences, wherein the polypeptide (n) has a function equivalent to that of said polypeptide (m).~~

10~~11~~. The method according to **any one of** claims **1 to 9** ~~10~~, wherein the fibronectin fragment has a cell adhesion activity and/or a heparin binding activity.

~~12. The method according to claim 10, wherein the fibronectin fragment is at least one polypeptide selected from the group consisting of polypeptides having any one of the amino acid sequences shown in SEQ ID NOs: 9 to 20 and 25 of Sequence Listing.~~

11~~13~~. The method according to claim 1 which is carried out in a cell culture equipment, wherein the method satisfies the conditions of:

(a) a ratio of the number of cells to a culture area in the cell culture equipment at initiation of culture being 1 cell/cm^2 to 5 X 10^5 cells/cm^2 ; and/or

(b) a concentration of cells in a medium at initiation of culture being 1 cell/mL to 5 X 10^5 cells/mL.

12~~14~~. The method according to claim 11~~13~~, wherein the method does not require a step of diluting a cell culture solution.

13~~15~~. The method according to claim 1, wherein the method comprises carrying out ~~at least any one of induction, maintenance and~~ **an** expansion of ~~a~~ cytotoxic lymphocytes in the presence of **the** fibronectin~~, a~~ fragment ~~thereof~~ or a mixture thereof in a cell culture equipment containing a medium, wherein the method comprises at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment, and wherein the culture conditions immediately after at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment satisfy the conditions of:

(c) a concentration of cells in the cell culture solution being 2 X 10^5 cells/mL to 1 X 10^8 cells/mL; or

(d) a ratio of the number of cells in the cell culture solution to a culture area in the cell culture equipment being 1 X 10^5 cells/cm^2 to 1 X 10^8 cells/cm^2 .

14~~16~~. The method according to claim 1, wherein the method comprises carrying out ~~at least any one of induction, maintenance and~~ **an** expansion of ~~a~~ cytotoxic lymphocytes in the presence of **the** fibronectin~~, a~~ fragment ~~thereof~~ or a mixture thereof in a cell culture equipment containing a medium, wherein the method comprises at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment, and wherein a total concentration of serum and plasma in the medium immediately after at least one step of diluting the cell culture solution, step of exchanging the medium, or step of exchanging the cell culture equipment is same as that at initiation of the culture or lowered as compared to that at initiation

of the culture.

~~17. A cytotoxic lymphocyte obtained by the method as defined in any one of claims 1 to 16.~~

~~18. A medicament comprising as an effective ingredient the cytotoxic lymphocyte obtained by the method as defined in any one of claims 1 to 16.~~

15~~19~~. A medium for culturing ~~a~~ cytotoxic lymphocytes, characterized in that the medium comprises as an effective ingredient **the** fibronectin~~,~~ ~~a~~ fragment ~~thereof~~ or a mixture thereof, and that a total concentration of serum and plasma in the medium is 0% by volume or more and less than ~~45%~~ by volume.

16~~20~~. The method according to any one of claims 1 to 14~~16~~, further comprising a step of transducing a foreign gene into ~~a~~ cytotoxic lymphocytes.

17~~21~~. The method according to claim 16~~20~~, wherein the foreign gene is transduced using retrovirus, adenovirus, adeno-associated virus or simian virus.

18~~22~~. A polypeptide ~~having~~ **consisting of** the amino acid sequence (x) shown in SEQ ID NO: 25 of Sequence Listing or an amino acid sequence (y) having deletion, insertion, addition or substitution of **1-20**~~one or the plural number of~~ amino acid**(s)** in the amino acid sequence (x), wherein the polypeptide ~~having~~ **consisting of** the amino acid sequence (y) has a function equivalent to that of the amino acid sequence (x).

19~~23~~. A nucleic acid encoding the polypeptide of claim 18~~22~~.

20~~24~~. The nucleic acid according to claim 19~~23~~, wherein the nucleic acid ~~comprises~~ **is** (1) a DNA ~~comprising~~ **consisting of** the nucleotide sequence shown in SEQ ID NO: 26; **or** (2) a DNA ~~comprising~~ **consisting of** a nucleotide sequence having deletion, substitution, insertion or addition of **1-60**~~one~~

~~or the plural number of~~ nucleotide(s) in the nucleotide sequence shown in SEQ ID NO: 26, wherein the DNA encodes a polypeptide having a function equivalent to that of the polypeptide encoded by the DNA (1)~~; or (3) a DNA which hybridizes to a DNA comprising the nucleotide sequence shown in SEQ ID NO: 26 under stringent conditions, wherein the DNA encodes a polypeptide having a function equivalent to that of the polypeptide encoded by the DNA (1)~~.

CLUSTAL X (1.83) MULTIPLE SEQUENCE ALIGNMENT

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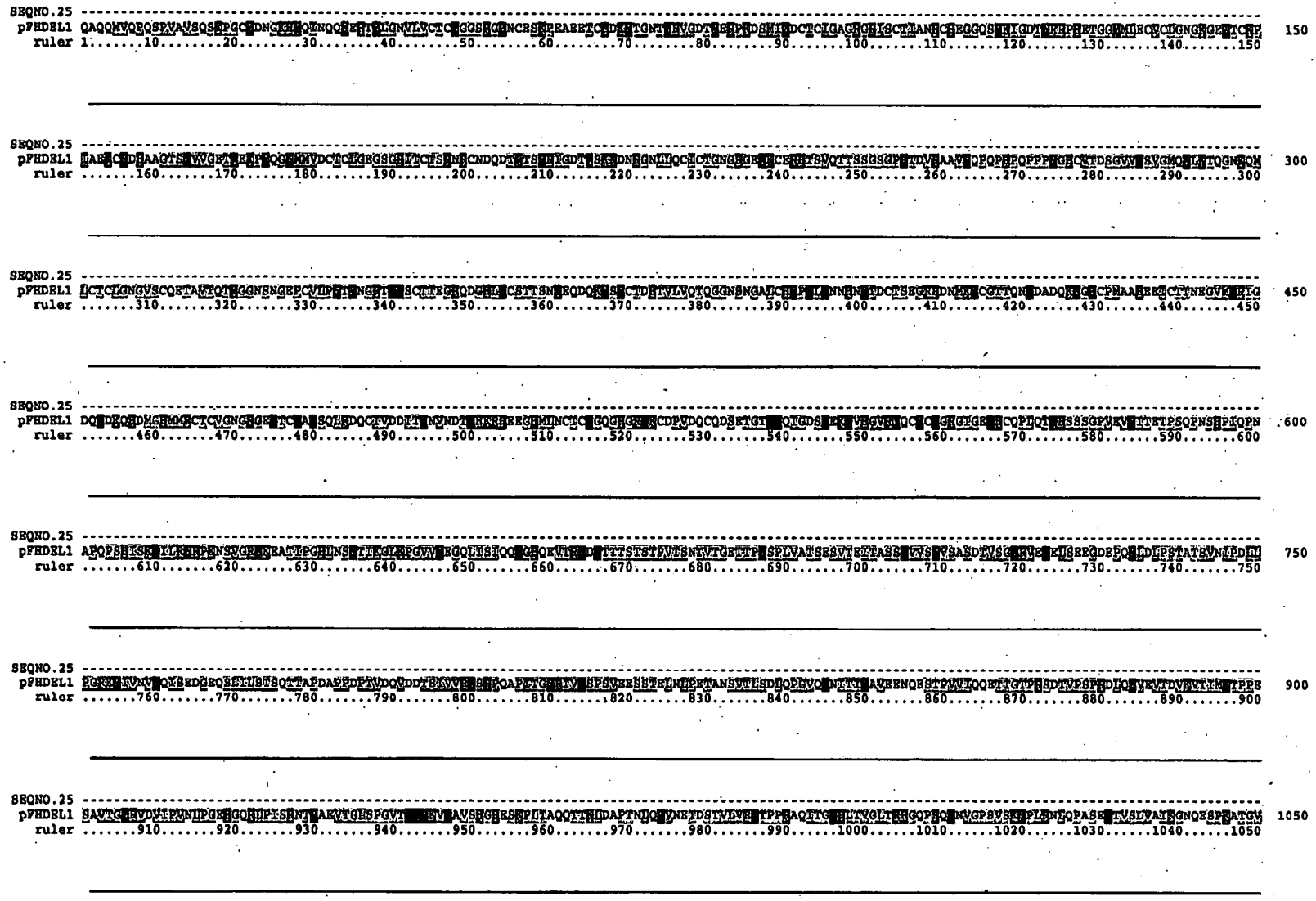

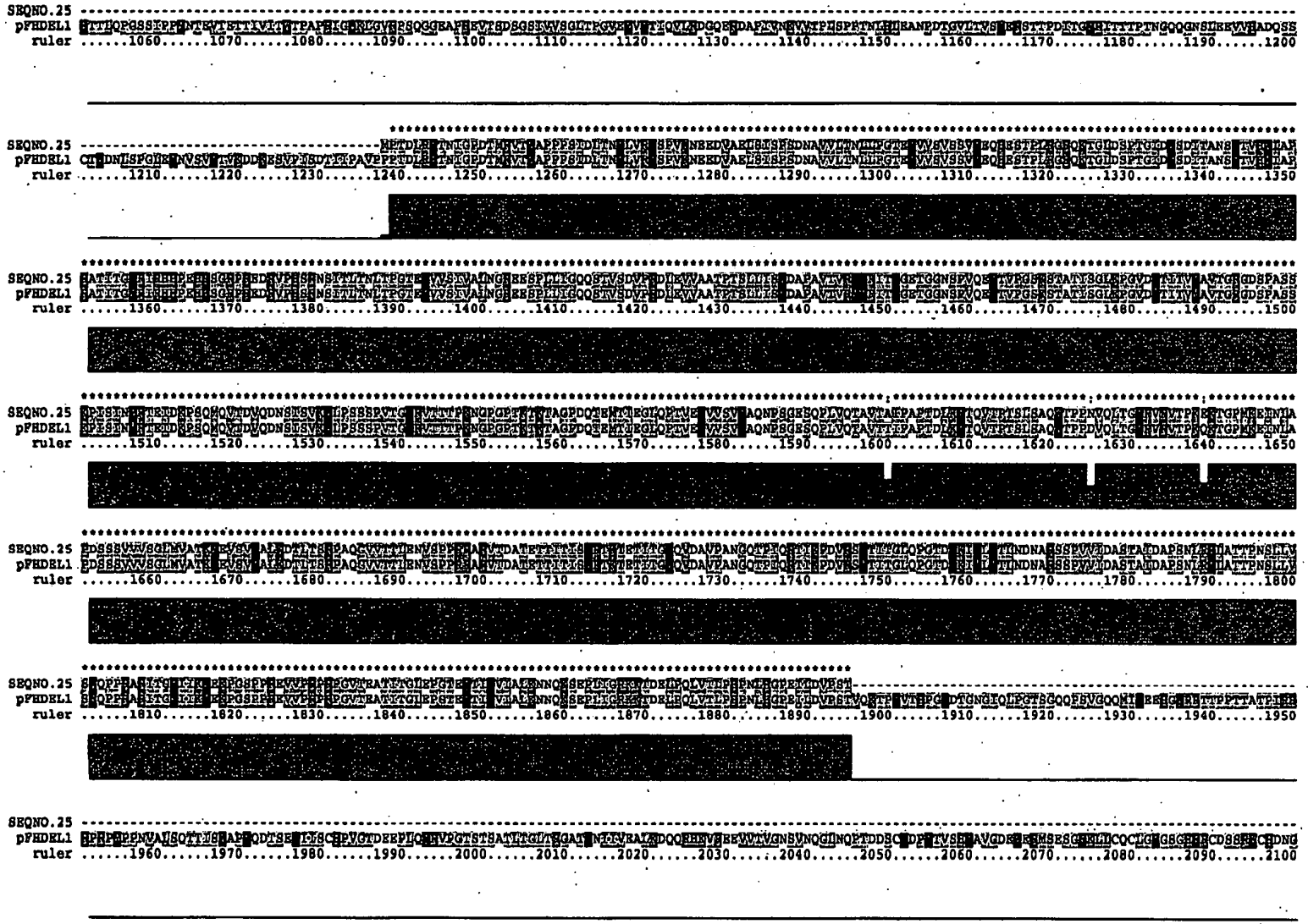

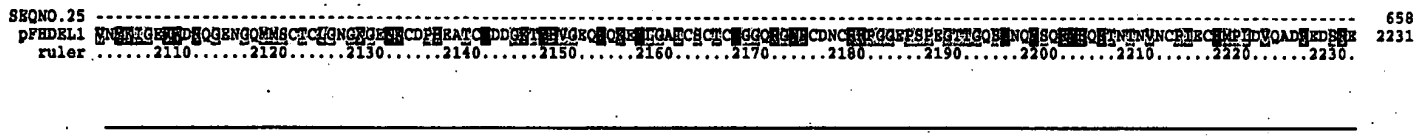
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CLUSTAL X (1.83) MULTIPLE SEQUENCE ALIGNMENT

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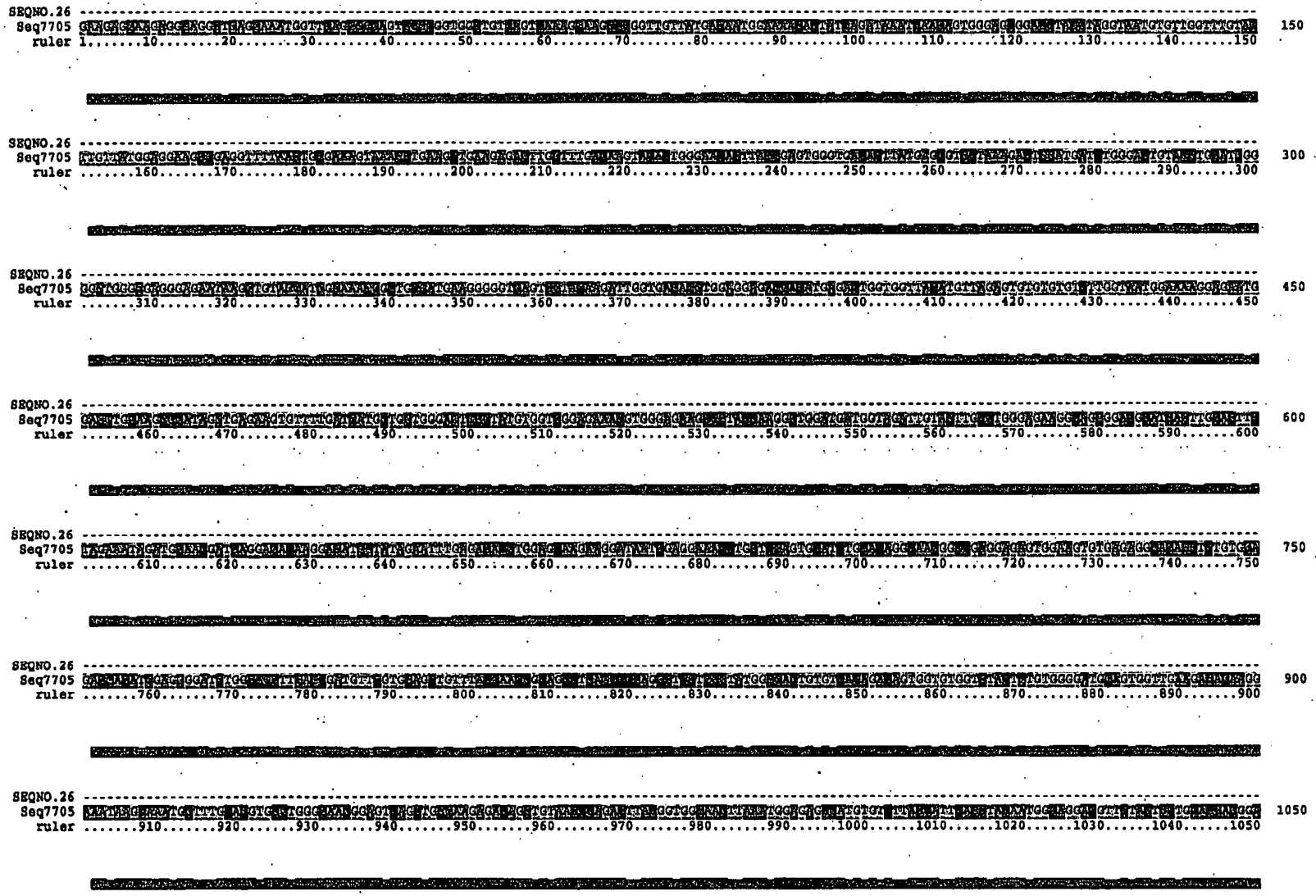

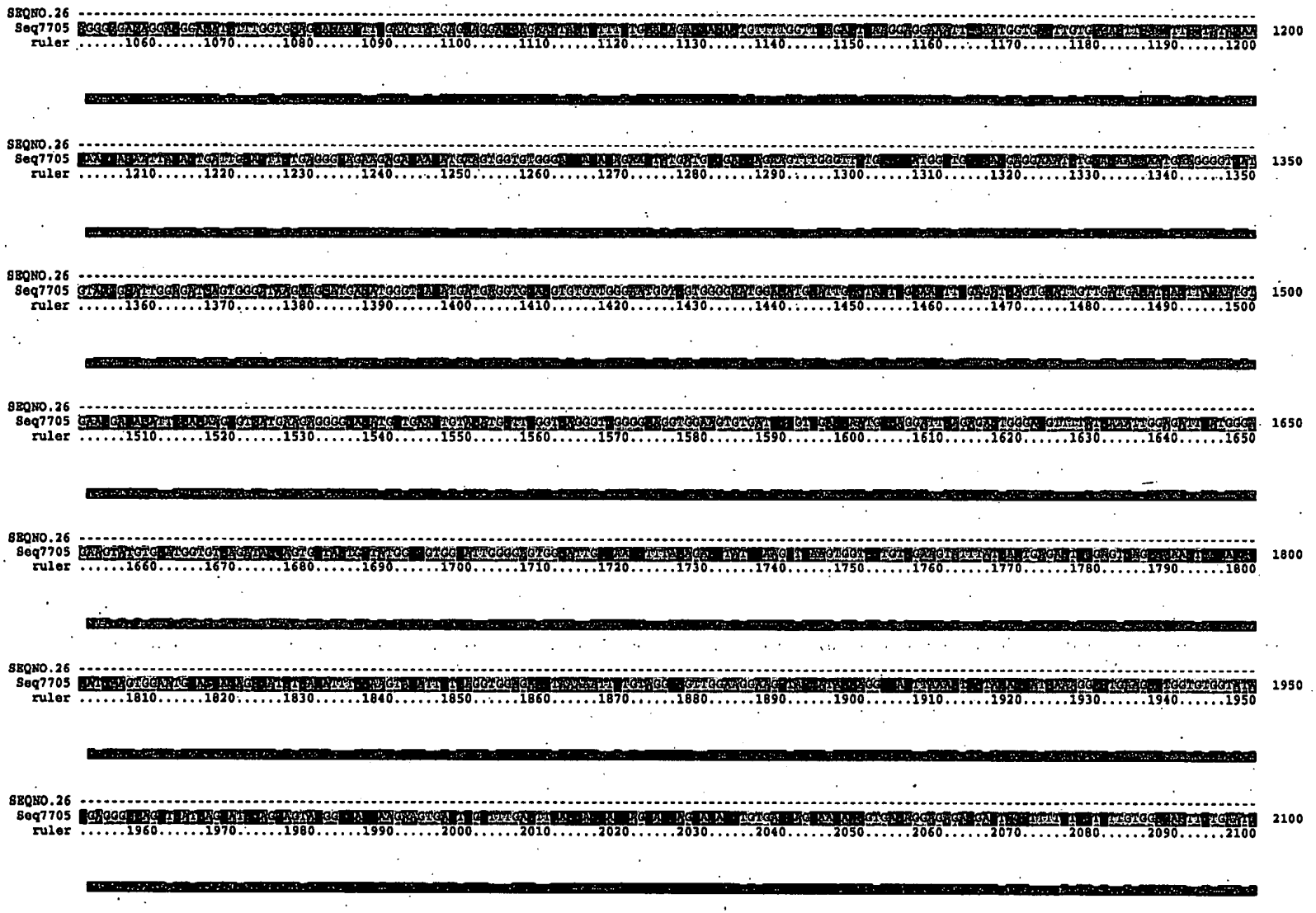
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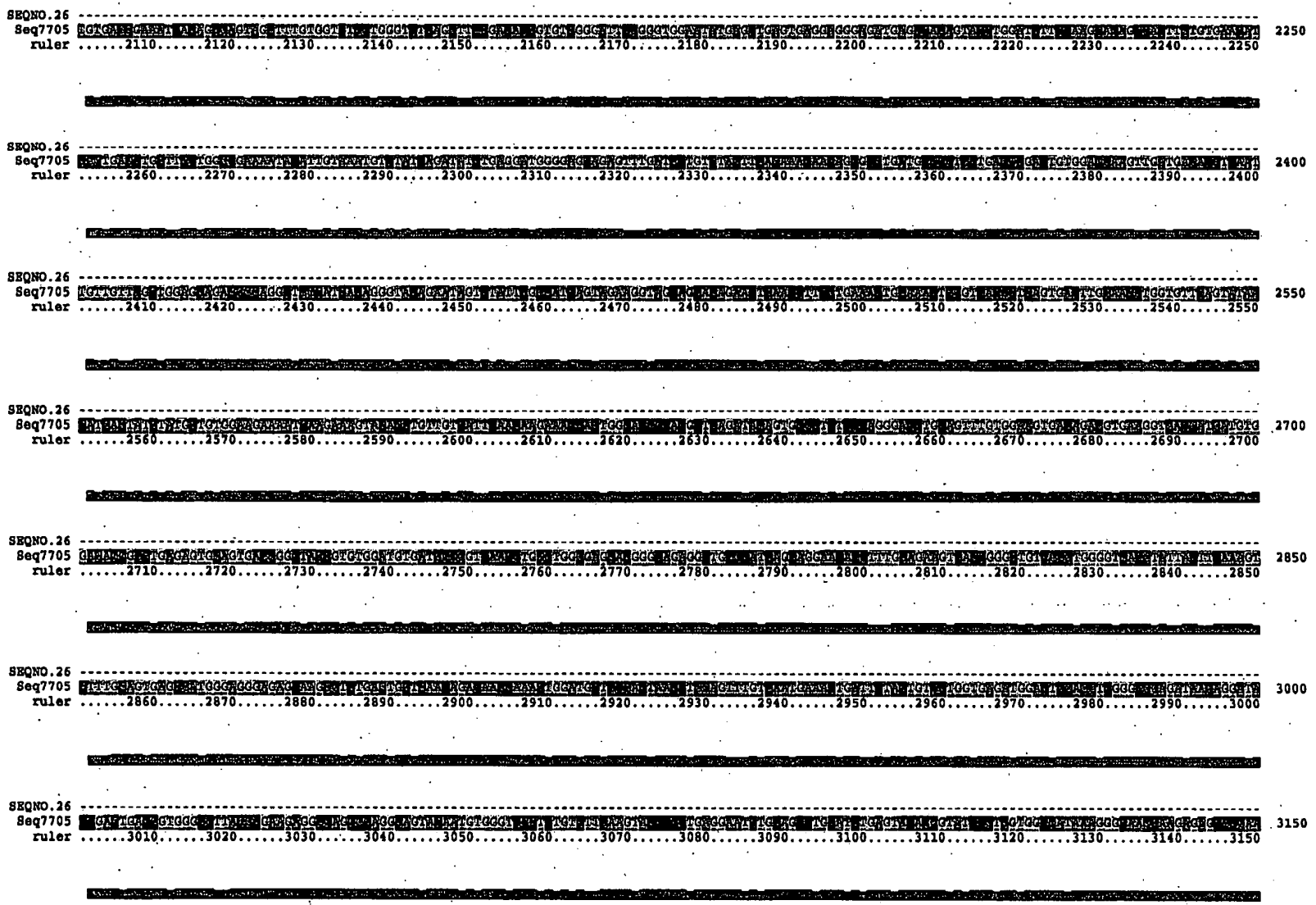

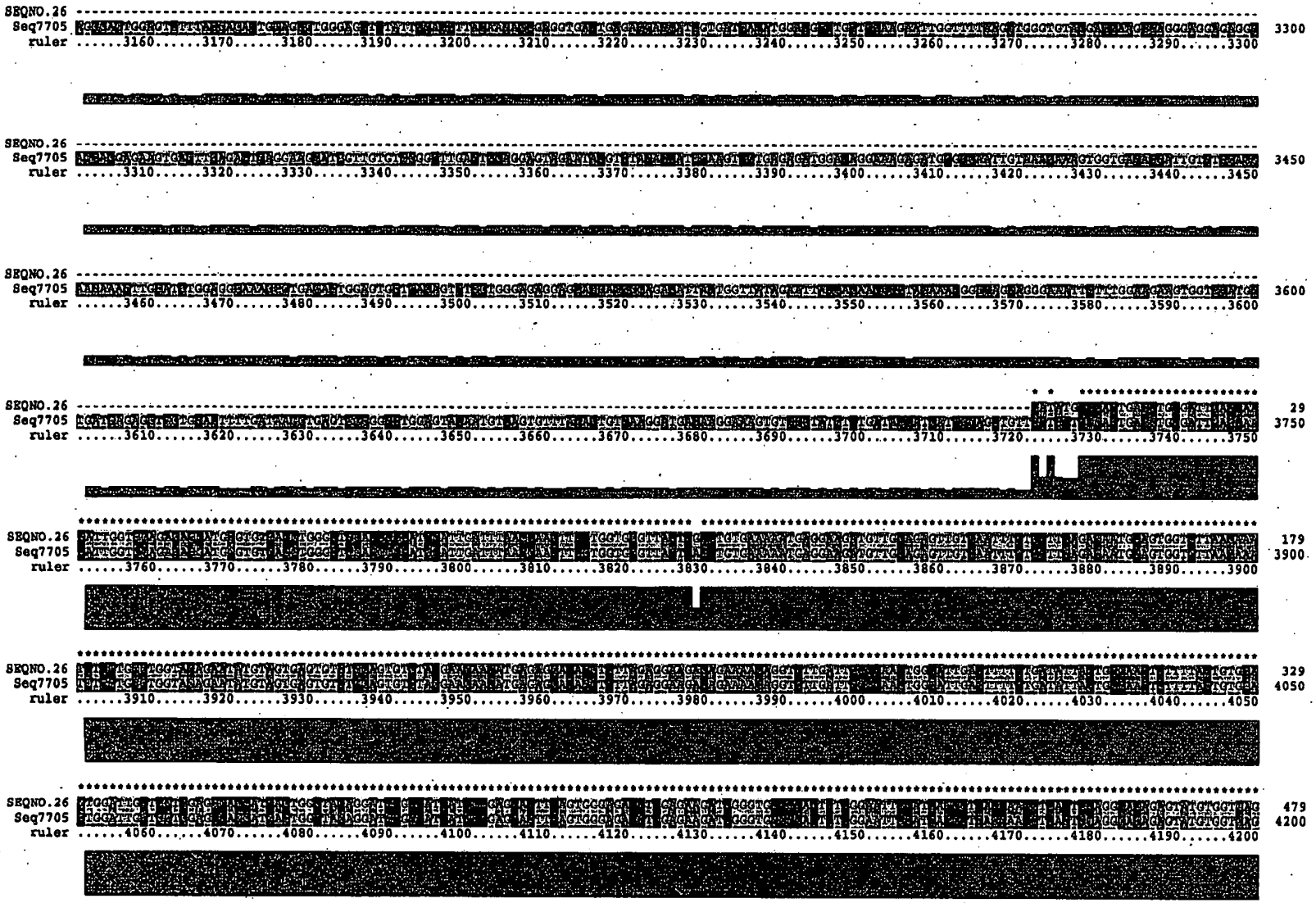
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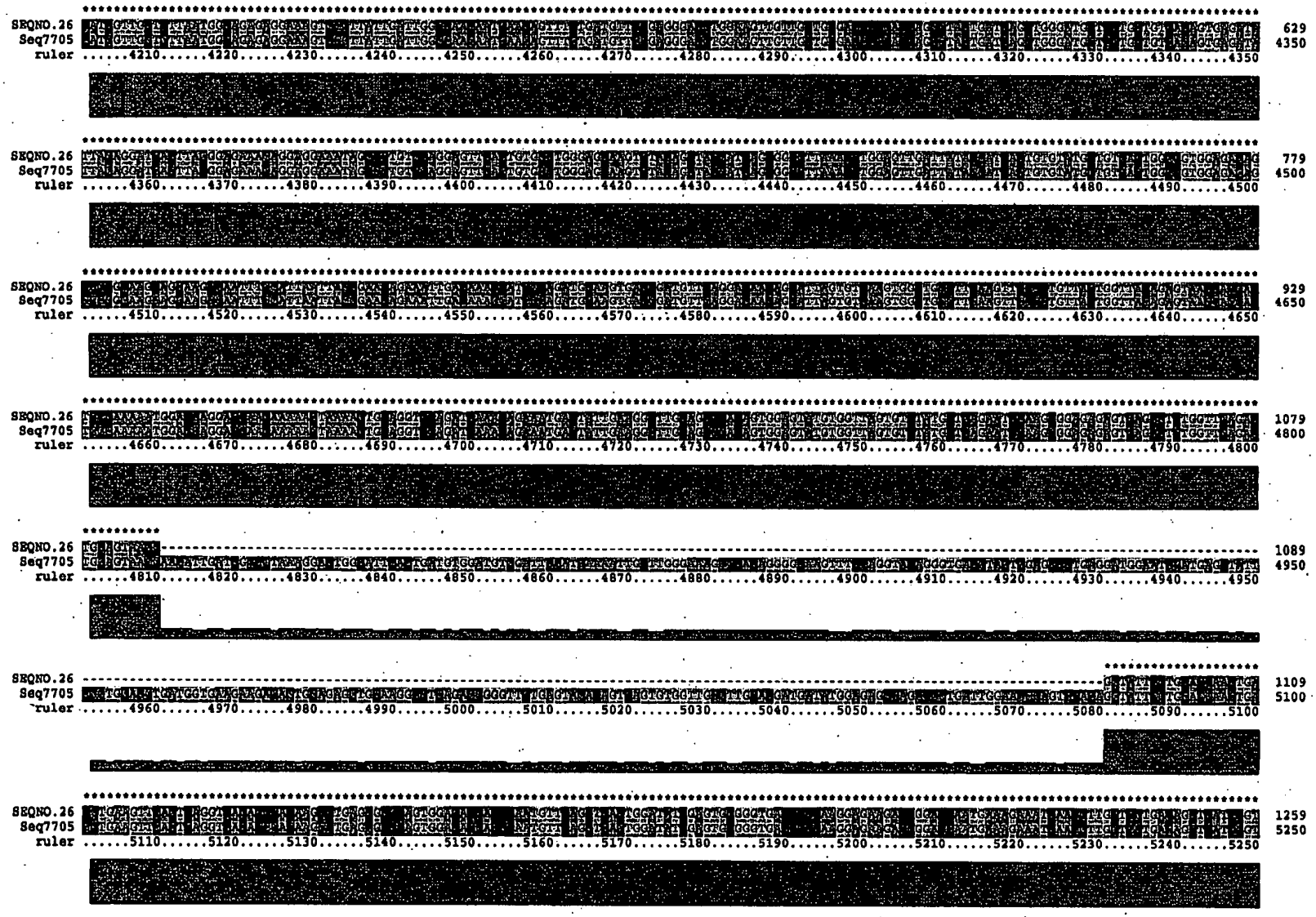
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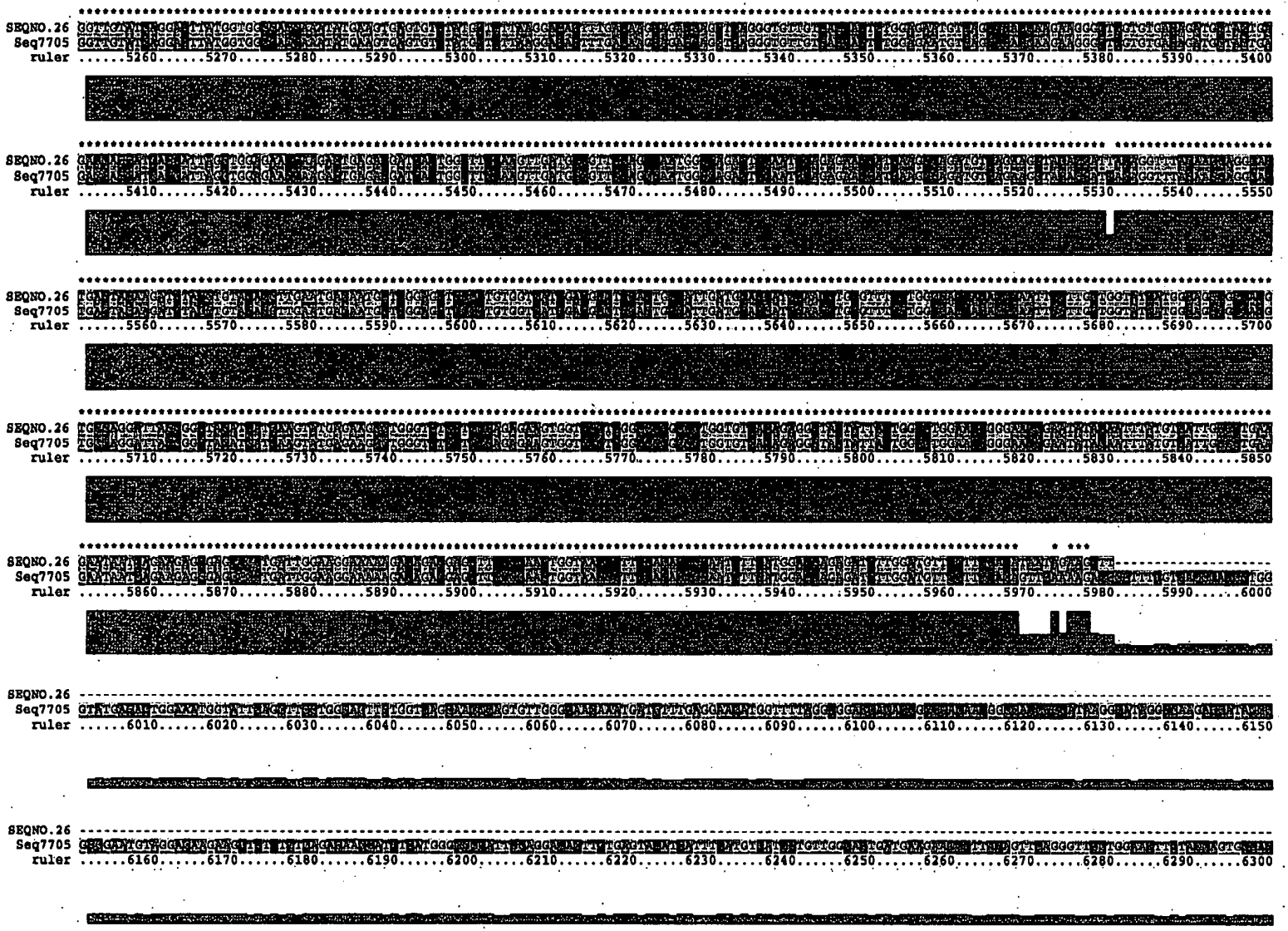
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CLUSTAL X (1.83) MULTIPLE SEQUENCE ALIGNMENT

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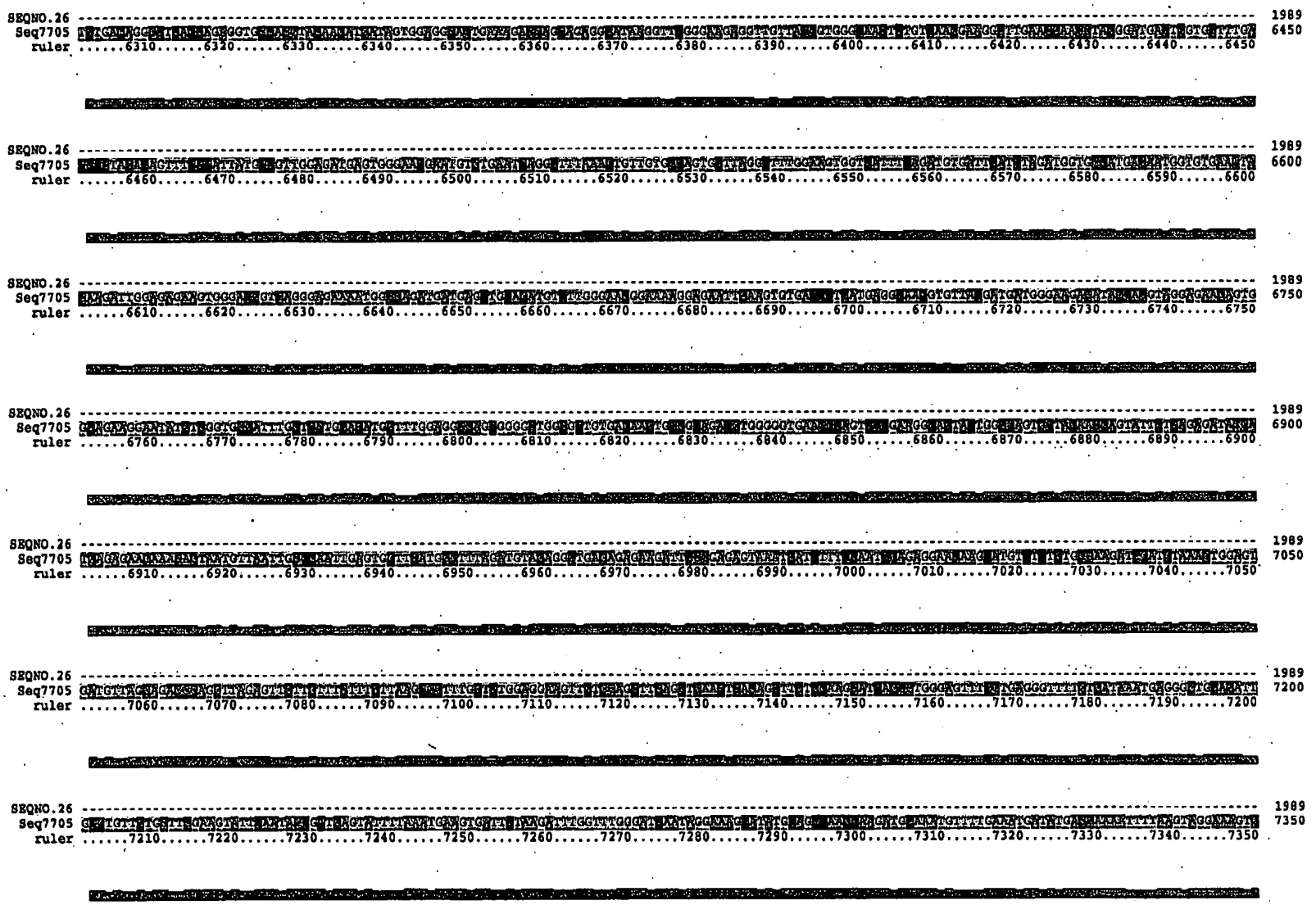

CLUSTAL X (1.83) MULTIPLE SEQUENCE ALIGNMENT

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